SCHOOL OF
CIVIL ENGINEERING

INDIANA
DEPARTMENT OF TRANSPORTATION

JOINT HIGHWAY RESEARCH PROJECT

FHWA/IN/JHRP-95/13
Final Report

BIOREMEDIATION TREATABILITY STUDIES FOR SOILS CONTAINING HERBICIDES, CHEMICALS, AND PETROLEUM PRODUCTS

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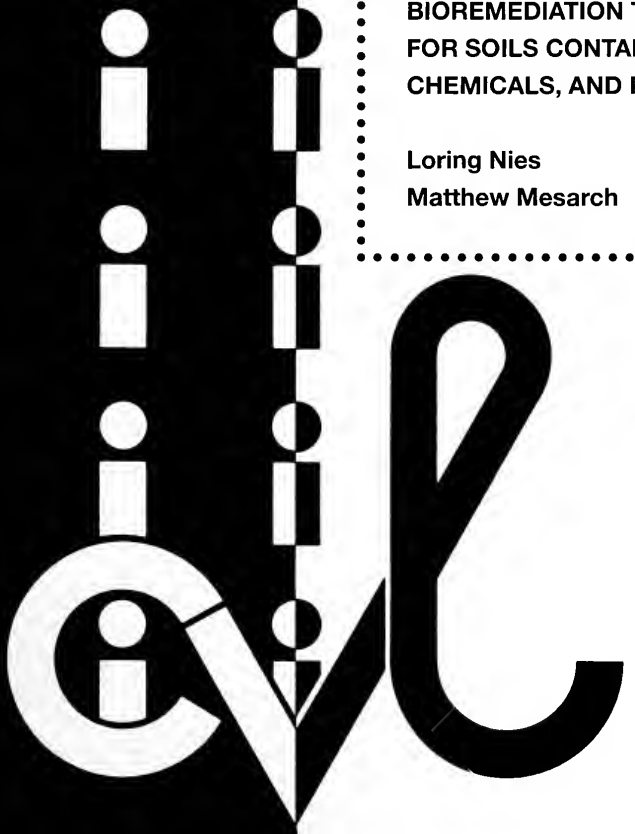

PURDUE UNIVERSITY

Final Report

BIOREMEDIATION TREATABILITY STUDIES FOR SOILS CONTAINING HERBICIDES, CHEMICALS AND PETROLEUM PRODUCTS

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Project No: C-36-68A
File: 4-7-1

Prepared in cooperation with the
U.S. Department of Transportation
Federal Highway Administration

Purdue University
West Lafayette, IN 47907
September 16, 1996

TECHNICAL REPORT STANDARD TITLE PAGE

1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
FHWA/IN/JHRP-95/13		

4. Title and Subtitle	5. Report Date
Bioremediation Treatability Studies for Soils Containing Herbicides, Chemicals, and Petroleum Products	September 16, 1996
	6. Performing Organization Code

7. Author(s)	8. Performing Organization Report No.
Loring Nies and Matthew Mesarch	FHWA/IN/JHRP-95/13

9. Performing Organization Name and Address	10. Work Unit No.
Joint Highway Research Project Civil Engineering Building Purdue University West Lafayette, Indiana 47907-1284	11. Contract or Grant No. HPR-2008

12. Sponsoring Agency Name and Address	13. Type of Report and Period Covered
Indiana Department of Transportation State Office Building 100 North Senate Avenue Indianapolis, IN 46204	Final Report
	14. Sponsoring Agency Code

15. Supplementary Notes

Prepared in cooperation with the Indiana Department of Highways and Federal Highway Administration.

16. Abstract

Leaking underground storage tanks are widespread throughout the United States. It is estimated that there are 1.4 million underground gasoline storage tanks in the United States, with as many as 75,000 to 100,000 that may be leaking. In Indiana alone, more than 3,500 of the 15,000 registered underground storage tank facilities have reported leaks. Conventional remediation methods often involve pump-and-treat schemes for contaminated water, and excavation and burial of contaminated soil in hazardous waste landfills. These methods increase the risk of exposure to pollutants for workers and local residents. Furthermore, these methods merely involve the transfer of pollutants from one environmental compartment to another, and are rather costly. Bioremediation is another method available for the restoration of contaminated sites. Advantages of bioremediation include competitive cost, pollutant destruction, and minimal environmental disturbance. By biodegrading organic pollutants on site, exposure to pollutants is minimized and costs are reduced. Bioremediation can potentially be an effective, low-cost, and terminal solution for remediation of sites contaminated with organic pollutants. The goal of bioremediation is to accelerate the biodegradation rates of naturally occurring microorganisms that utilize organic pollutants as a food source. The overall objective of this study was to determine whether bioremediation is a feasible treatment option for contaminated INDOT soils. All INDOT soils tested had three things in common. First, a thriving heterotrophic microbial population existed. Second, bacteria capable of degrading benzoate (a toluene surrogate) were present in all soils. Finally, toluene biodegradation in all soils indicated indigenous populations with the catabolic capabilities to remediate petroleum hydrocarbons. This study provides some evidence that physical, rather than microbial, parameters control biological processes in soil. Based on these results as a whole, we conclude that in-situ bioremediation of petroleum hydrocarbons is a treatment option which should have increased utilization.

17. Key Words	18. Distribution Statement
bioremediation, petroleum, gasoline, LUST, leaking underground storage tanks, soil remediation, groundwater.	No restrictions. This document is available to the public through the National Technical Information Service, Virginia, 22161

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of Pages	22. Price
Unclassified	Unclassified	38	

Form DOT F 1700.7 (8-69)

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Abstract

Many organic chemicals have known human and environmental health hazards. At sites where chemicals have historically been stored, mixed and transferred, spills have often occurred. A primary route for human exposure to hazardous chemicals is by drinking groundwater which has been contaminated due to spills onto soil (Wilson et al., 1986) Due to the potential human health risks associated with exposure to hazardous chemicals, it is desirable to restore contaminated sites by the removal of pollutants.

Conventional remediation methods often involve pump-and-treat schemes for contaminated water, and excavation and burial of contaminated soil in hazardous waste landfills. These methods increase the risk of exposure to pollutants for workers and local residents. Furthermore, these methods merely involve the transfer of pollutants from one environmental compartment to another, and are rather costly. Bioremediation is another method available for the restoration of contaminated sites. Advantages of bioremediation include competitive costs, pollutant destruction and minimal environmental disturbance. By biodegrading organic pollutants on site, exposure to pollutants is minimized and costs are reduced. One of the disadvantages of bioremediation is performance uncertainties in low permeability soils. Despite these potential reliability problems, bioremediation can potentially be an effective, low-cost and terminal solution for remediation of sites contaminated with organic pollutants. Many organic compounds, petroleum products in particular, are known to be biodegraded by indigenous microorganisms. The goal of bioremediation is to accelerate the biodegradation rates of naturally occurring microorganisms that utilize organic pollutants as a food source.

An additional and important benefit of biodegradation as a remediation strategy is that pollutants are transformed into harmless products and not merely transferred to another environmental compartment (e.g. landfilling). Often the limiting factor controlling natural biodegradation processes are easily manipulated environmental parameters such as moisture, pH, oxygen

availability and nutrient limitations (N, P). The manipulation of environmental conditions to accelerate naturally occurring biodegradation processes is bioremediation. Several carefully controlled successful field demonstrations of bioremediation have taken place in recent years.

Introduction

Leaking underground storage tanks (LUST's) are widespread throughout the United States. It is estimated that there are 1.4 million underground gasoline storage tanks in the United States, with as many as 75,000 to 100,000 that may be leaking (Hutchins, 1991). In Indiana alone more than 3,500 of the 15,000 registered underground storage tank (UST) facilities have reported leaks (IDEM, 1993).

Numerous models have been developed to assess the risk of exposure to petroleum constituents due to environmental transport. Modeling petroleum transport in the unsaturated zone is more difficult than in the saturated zone. In the saturated zone, soluble constituents of petroleum (e.g. BTEX) are dissolved in the aqueous phase. The soluble constituents move with the bulk fluid, although their overall transport velocity is retarded relative to the groundwater velocity by interactions (sorption/desorption) with soil. Migration of BTEX from the source can be very significant (hundreds of meters). The volume fraction of petroleum contaminants in the bulk fluid phase is typically very small ($<<<$ 0.01 %).

In the unsaturated zone, without the driving force of a hydraulic gradient transport is controlled by contaminant-soil interactions (sorption), capillary forces, and gravitational forces. Sorption and capillary forces tend to balance gravitational forces, and therefore, contaminant migration can be relatively insignificant for small releases (small relative to the bulk soil volume). However, infiltration of rainwater through the unsaturated zone will periodically provide a hydraulic driving force and will result in spreading of petroleum constituents downward. Variables such as the amount of infiltration water, soil hydraulic conductivity, and soil organic content, as well as the volume of contaminant, released will significantly influence transport in the unsaturated zone. Overall however, without a constant hydraulic driving force contaminant migration in the unsaturated zone is typically much less significant than in the saturated zone.

Therefore, the primary hazard posed by LUST's is the possible contamination of groundwater, which comprises most of our drinking water supply. Components of gasoline such as the BTX compounds (benzene, toluene, and the xylene isomers) can cause health problems via inhalation, ingestion, and in some cases, skin contact. Benzene inhalation can cause depression of central nervous system activity and in extreme cases, respiratory failure. Toluene and the xylenes can also depress central nervous system activities (Dean 1978). Long-term exposure to benzene can lead to leukemia while long-term exposure to toluene and the xylenes can again lead to further central nervous system problems. Toluene and the xylenes have been reported to enhance skin carcinogenesis (Dean, 1978). With the number and severity of health problems the BTX compounds pose, it is obvious that their exposure to humans be minimized and that polluted groundwater and soil be remediated. Due to potential health hazards associated with human exposure to BTX compounds, regulations have been established which require that sites at which petroleum contamination exists must be remediated.

Leaded fuel products (e.g. leaded gasoline) contain up to 800 ppm tetraethyllead or tetramethyllead, thus, it is useful to consider the potential hazards arising from lead contamination. The concentration of lead in soil resulting from the release of leaded fuel typically will not reach levels which would require remediation due to lead contamination. Often, the lead input from release of leaded fuel is insignificant when compared to background levels of lead. This however depends entirely on the volume of fuel released. For example, a soil contaminated with leaded fuel at a concentration of 100 ppm TPH would have approximately 60 ppb lead originating from the fuel.

Bacterial Metabolism

Cell Structure

In order to understand how bacteria can degrade pollutants like toluene it is important to understand the basic components of a bacterial cell. A single bacterial cell, or bacterium, is

protected from its environment by a rigid cell wall. This wall both protects the cell's contents as well as provides structural rigidity to the cell (Brock, 1994). Past the cell wall lies the cell membrane, also known as the cytoplasmic membrane (Brock, 1994). About 8 nm thick, this membrane holds the cell's contents together (Brock, 1994). It is less permeable than the cell wall, preventing even hydrogen and hydroxyl ions from traversing its width. Cytoplasmic membranes are composed of phospholipids arranged in a bilayer so that the inner portion of the membrane is composed of fatty acids and is very hydrophobic (Brock, 1994). The inner and outer surfaces of the membrane are hydrophilic, they are composed of glycerols and phosphate groups (Brock, 1994). Within the membrane reside electron transport proteins, vital energy producing enzymes, and various other transport proteins (Brock, 1994). It is across this membrane that all nutrients are brought into the cell and all waste products are excreted from it by specific transport proteins. Within the membrane is the cytoplasm. The cytoplasm is a conglomerate of enzymes, salts, sugars, vitamins, amino acids and the nucleoid. The nucleoid of a cell consists of a single DNA strand which contains all of a cell's genetic information, it is the blueprint of a cell (Brock, 1994).

Bioremediation of Petroleum Hydrocarbons

Bioremediation Theory

Bioremediation, simply put, is the use of microorganisms (usually bacteria) to convert hazardous pollutants into less harmful ones. Products of bioremediation are energy, which is used by the bacteria in biosynthesis reactions to create new cells, and carbon dioxide (National Research Council, 1993). The pollutant is a source of both electrons and carbon for bacteria (National Research Council, 1993). The electrons are transferred to an electron acceptor (oxygen), a process which is harnessed to release energy. The carbon is used for cellular synthesis. Often two types of bioremediation are mentioned: intrinsic bioremediation and engineered bioremediation. Intrinsic bioremediation occurs without human intervention while engineered bioremediation relies on manipulation of some property of the site (e.g. supply of oxygen or nutrients) in order to accelerate biodegradation rates (National Research Council, 1993).

Advantages and Disadvantages of Bioremediation

Bioremediation is a promising new remediation technique that has the potential to completely mineralize pollutants to carbon dioxide. Because of this advantage, bioremediation is often called a terminal solution to pollutants. By mineralizing pollutants long-term risk to interested parties is eliminated and liabilities are mitigated (Cookson, 1995). Such would not be the case if, for example, a large amount of gasoline-contaminated soil were to be excavated and landfilled, a practice which has been commonly utilized. Landfilling does not eliminate the contamination; instead it is merely transferred to a new location. The liabilities still exist for the waste and new ones arise as excavation and transportation costs and liabilities are accrued (Cookson, 1995). If that same site were to undergo in-situ bioremediation not only could the polluted soil be cleaned up, it would be done so at a cheaper cost than landfilling (Cookson, 1995). In addition, bioremediation tends to be non-intrusive, thereby lessening physical disruption to the site (Cookson, 1995).

Bacterial Requirements

Bacteria have a number of nutritional requirements that must be met in order to thrive. Most important is a carbon source, which is ideally the hydrocarbon pollutant, and an electron acceptor, which is usually oxygen. Suitable moisture conditions must also exist (Cookson, 1995). Bacteria are 70 to 90 percent water by weight, which illustrates the importance of an ample water supply (Brock, 1994). Apart from this, water facilitates many important transport processes for bacteria. First, water transports nutrients and electron acceptors like oxygen directly to cells that have no motile capabilities (Tate, 1995). Conversely, motile cells use water as a medium in which to move about in. Water also transports waste products away from cells and participates in enzyme mediated reactions such as hydrolysis (Tate, 1995). Ultimately a sufficient supply of water is critical to the survivability of a healthy microbial population. Luckily soil bacteria are essentially

bathed in water, filling voids between soil particles or existing as a film several micrometers thick (Tate, 1995). Other nutrients such as nitrogen and phosphorus are also necessary (Cookson, 1995). A typical cell contains 50% carbon, 14% nitrogen, 3% phosphorus, and trace amounts of other elements such as potassium, sulfur, iron, and other metals (National Research Council, 1993). Logically, to make new cells, bacteria need nutrients. Often nutrients already exist in soils: nitrogen can be fixed by some bacteria, dead plant and animal matter as well as inorganic minerals can supply other nutritional requirements (Norris, 1994). Whether an ample supply of nutrients exists is a site specific issue. A commonly applied rule of thumb is that a ratio of 100:10:1 carbon:nitrogen:phosphorus is required to completely convert hydrocarbons to cell material. However, the amounts of nitrogen and phosphorus actually required is usually less since carbon dioxide will also form as a biodegradation product (Norris, 1994).

Soil type is an extremely important parameter to consider as well. Sandy and gravely soils are better candidates for bioremediation as they allow for easier transport of water, nutrients, and oxygen (Norris, 1994). A final requirement that can not be overlooked is the presence of organisms capable of degrading the pollutant (Cookson, 1995). In the case of petroleum hydrocarbons such as gasoline, this is not usually a problem. In fact, soil microbes are so capable of degrading petroleum hydrocarbons that seeding a soil contaminated by petroleum with lab-tested petroleum-degrading microorganisms is often a futile task. The introduced microorganisms are not capable of competing with the indigenous petroleum-degrading microorganisms which are acclimated to that environment (Leahy, 1990). Optimization of the aforementioned requirements can lead to rapid degradation of pollutants in the environment.

Remediation of Petroleum-Contaminated Sites

Petroleum hydrocarbons are biodegradable. Lighter, more soluble compounds degrade more readily than less soluble compounds (Norris, 1994). The first in-situ bioremediation system was installed over 20 years ago in Pennsylvania to remediate an oil pipeline spill (National

Research Council, 1993). Bioremediation has been used on a large scale to mitigate the effects of the Exxon Valdez spill in Prince William Sound, Alaska (Button, 1992; Venosa, 1992) and the release of oil during the Persian Gulf War (Fayad, 1992; Hasan, 1994). Bioremediation on a smaller scale, however, is more common and certainly no less worthwhile. Reduction of the toxicity of soils contaminated by jet fuel, heating oil, and diesel oil were achieved by nutrient addition and tilling of the soil (Wang, 1990). Tilling alone to promote soil aeration reduced soil toxicity as well (Wang, 1990).

Bacteria can be quite resilient, withstanding high concentrations of contaminants. Bacteria were reported to be in excess of 10^8 cells per gram of dry hazardous waste-contaminated soil from Northern Indiana where toluene concentrations were reported to be 80,000 ppm toluene (Ross, 1989). Recently a strain of Pseudomonas putida was isolated that could survive in toluene emulsions of up to 50% by volume, or approximately 435,000 ppm (Inoue, 1989). Obviously, petroleum (and therefore, toluene) degrading bacteria are ubiquitous in nature and can be capable of withstanding extremely high concentrations of pollutants in some instances. Measuring rates of petroleum degradation are complicated by the fact that petroleum is composed of many different compounds, each being consumed at different rates (Song, 1990).

Toluene Degradation

Microbial degradation of toluene, like any other pollutant, proceeds along a number of well-established pathways. Aerobically bacteria use dioxygenase and monooxygenase enzymes to insert oxygen into the structure of toluene as well as to cleave the aromatic ring. One such pathway involves an initial attack on the methyl ($-CH_3$) group on the aromatic ring, oxidizing it to benzoic acid (Smith, 1990). Next a dioxygenase enzyme inserts a molecule of oxygen into the aromatic ring, breaking a double bond, making benzoate-1,2-dihydrodiol (Smith, 1990). Finally benzoate diol dehydrogenase transforms the compound to catechol.

Degradation of toluene can also occur via initial attack on the aromatic ring. First the dioxygenase enzyme inserts the oxygen into the aromatic ring structure, forming cis-1,2-dihydroxy-2,3-dihydrotoluene (Smith, 1990). Next the hydrogens at the 2,3 positions are removed forming 3-methylcatechol.

Regardless of whether the initial attack is on the methyl group or on the aromatic ring, degradation of toluene, as well as other aromatic compounds, typically converges at a catechol-like intermediate (Gottschalk, 1986). Another common intermediate is protocatechuate (Gottschalk, 1986).

Once a catechol intermediate is formed cleavage of the aromatic ring may occur. Two pathways of ring cleavage are observed: ortho cleavage and meta cleavage (Gottschalk, 1986). In ortho cleavage, a dioxygenase enzyme cleaves the aromatic ring between the two hydroxyl (-OH) groups to form cis, cis-muconate while in meta cleavage, the ring is cleaved adjacent to the hydroxyl-bearing carbons forming 2-hydroxymuconic semialdehyde (Gottschalk, 1986). Once ring cleavage has occurred, further breakdown of the compound can begin.

Xylenes, if not utilized as primary substrates, may be cometabolized by organisms with dioxygenase enzymes. The catechol intermediates formed from xylene cometabolism may not be degraded further.

Tricarboxylic Acid Cycle

Once the cleavage of the aromatic ring has occurred, further degradation to compounds such as pyruvate, a 3 carbon compound, occurs. In the TCA cycle pyruvate undergoes a series of enzyme mediated oxidations to form carbon dioxide. Bacteria use the TCA cycle to break down compounds into carbon dioxide, resulting in the formation of reduced coenzymes such as NADH and NAD(P)H. These reduced coenzymes are then used to make energy in the form of adenosine

triphosphate (ATP), in a process known as electron transport phosphorylation (Brock, 1994). ATP is used to drive biosynthesis reactions involved in creating vital cellular components. A second function of the TCA cycle is the production of intermediate compounds (e.g. succinate) which are be used as building blocks for cellular components (Gottschalk, 1986). The TCA cycle provides a vital link between physical breakdown of pollutants such as toluene and cellular energy production.

Problem Statement

Soil contamination by hazardous pollutants may exist at some Indiana Department of Transportation facilities. Potential pollutants are petroleum products, herbicides and solvents. Traditional site remediation and disposal methods are often prohibitively expensive, therefore, the feasibility of more cost-effective remediation methods must be considered.

Costs for landfilling are at least $65 per cubic yard of material. Additional fees for excavating and hauling the soil, sampling required by the landfill, permits required, and decontamination of all equipment may increase this cost estimate. Estimates for bioremediation are as low as $35 per cubic yard and no permits are required unless groundwater is to be remediated (IDEM, 1993). Any liability for the contaminated soil remains on-site until remediation is complete.

Objectives

The overall objective of this study was to determine whether bioremediation is a feasible treatment option for contaminated INDOT soils. Bioremediation is simply the utilization of microorganisms to treat contaminants. The byproducts of bioremediation, or biotreatment, are simply the growth of new microorganisms and CO_2. In order to determine the capacity of the indigenous soil microorganisms to degrade the pollutants the type and level of contaminants in the soil must first be characterized. Next a healthy indigenous population of microorganisms must exist. Finally the extent to which these microorganisms can degrade these pollutants must be established.

Objective 1.

Characterize the type and level of contamination. Soil samples will be analyzed to determine the identification and concentration of pollutants present.

Objective 2.

Characterize the presence of indigenous pollutant degrading microorganisms. In the laboratory, confirm the presence of specific microorganisms capable of degrading organic pollutants in each sample.

Objective 3.

Determine the extent to which oxygen and nutrient additions are required to promote biodegradation. To determine the feasibility of implementing in situ bioremediation, factors potentially limiting intrinsic biodegradation such as nutrient limitations, will be determined.

Work Plan

Objective 1. Characterize the type and level of contamination.

Soil samples will be extracted and analyzed for contamination. Both non-polar solvent extractable components and polar aqueous (acid and base) extractable fractions will be examined. Analysis will be done using gas chromatography and liquid chromatography. Compound identification will be accomplished with UV-VIS spectrometry and mass spectrometry. If available, site records will be utilized to aid in the determination of the identification of pollutants.

Objective 2. Characterize the presence of indigenous pollutant degrading microorganisms. A critical step in determining the feasibility of bioremediation is the demonstration that

microorganisms capable of biodegrading specific pollutants exist at each contaminated site. Based on the results from objective 1, microbial enrichment and isolation procedures will be conducted. For example, if it is determined that toluene (a component in gasoline) or 2,4-D (an herbicide) is present in soil samples, conditions suitable for microorganisms capable of utilizing these compounds as growth substrates will be provided and pure cultures of microorganisms utilizing the pollutant of interest (e.g. toluene or 2,4-D) will be isolated.

Objective 3. Determine the extent to which oxygen and nutrient additions are required to promote biodegradation. The rate-limiting factors of pollutant biodegradation are typically oxygen availability or nitrogen and/or phosphorus deficiencies. Laboratory microcosms will be set-up to determine the effect of oxygen additions and nutrient supplements on the biodegradation of organic pollutants in INDOT soils. The disappearance of pollutants will be monitored over time, as well as the appearance of intermediate degradation products. Replicate samples and control samples will also be monitored.

Collection and Storage of Samples

Soil samples from INDOT facilities were collected by Farlow Environmental Engineers using hand sampling equipment. All samples were placed in one quart glass jars and field screened with a photoionization detector to provide a preliminary indication of petroleum hydrocarbon contamination. The inside of each lid was lined with aluminum foil to prevent sorption of volatile petroleum hydrocarbons. Upon their receipt at Purdue, all samples were stored in the dark at 4.5∞C. Samples were collected from INDOT facilities in Anderson, Bean Blossom, Dale, Hamlet, Hanna, Ridgeville, St. John, and Vincennes, Indiana. Two soil samples from each site were collected with the exception of Vincennes where three samples were collected.

Initial Headspace Tests

In addition to the preliminary photoionization screening of all samples by Farlow Environmental Engineers, headspace testing of all samples for volatile petroleum hydrocarbons was performed by gas chromatography. All soil samples were warmed overnight to room temperature. 500 ul of headspace was collected from each sample with a gas tight syringe by puncturing the foil covering on the jars. The headspace was then injected into the gas chromatograph.

All samples were analyzed with a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a flame ionization detector. Headspace injections were done manually into a cool on-column injection port. The column used was a 60-meter DB-1 (polydimethylsiloxane bonded phase) capillary column (0.53 mm ID and 1.5 um film thickness). Helium was used as a carrier gas at a pressure of 6 psi and a flow rate of 5.5 ml per minute. The injector temperature was initially set at 33∞C. This temperature was increased over the duration of the run to maintain an injector temperature 3∞C above the oven temperature for the entire run. The detector was maintained at a constant temperature of 210∞C. The GC oven was temperature programmed initially at 30∞C for 10 minutes, then the temperature was ramped at 1∞C per minute to 40∞C where it was held constant for one minute, then the temperature was ramped at 10∞C per minute to 160∞C. Finally, the temperature was ramped at 20∞C per minute to 260∞C and held constant for 5 minutes. All data was analyzed and collected using Hewlett Packard HP-CHEM software. Headspace samples were used merely as a positive or negative indicator of the presence of volatile petroleum hydrocarbons.

Extraction Procedure and Analysis

All soil samples were extracted with methanol to investigate whether nonvolatile petroleum hydrocarbons were present in the soil. Approximately 1 gram of soil (dry weight) was extracted with 10 ml methanol in glass vials with Teflon-lined rubber stoppers. These were manually shaken for 1 minute. The methanol was then drawn off with sterile glass disposable pipettes. All extracts were centrifuged in COREX® centrifuge tubes at 7000 rpm for 10 minutes on a Sorvall RC-5B Superspeed Refrigerated Centrifuge.

Extracts were analyzed via gas chromatography on the same instrument describe above. Liquid samples (2.5 ul) were injected using a Hewlett Packard 7673 Automatic Sampler. To ensure that no sample carryover existed between runs, methanol blanks were run at the beginning of all autosampler sequences as well as after every 3 or 4 sample runs. All extracts were injected in triplicate. Analysis was made by comparing the chromatograms of extracts against those of gasoline standards that are described below.

Gasoline Standards

A gasoline calibration curve was made using unleaded gasoline. This curve was used to calculate the concentration of petroleum hydrocarbons in the soil of all extracts. An 18,000 mg/l stock unleaded solution was made by diluting 250 ul unleaded into 9.75 ml methanol. 100 ul stock was then diluted into 9.9 ml methanol to make a 180 mg/l solution. 100 ul of this was further diluted into 9.9 ml methanol to make a 18.0 mg/l solution. Finally a 1.80 mg/l solution was made by diluting 10 ul of the 18.0 mg/l solution. All standards were injected in triplicate with an autosampler onto the GC described above.

Heterotrophic Plate Counts

Heterotrophic plate counts were performed to measure the viability of the indigenous microbial population. In this procedure 1 gram soil samples (dry weight) were extracted with a solution containing 90 ml sterile nanopure water and 10 ml phosphate buffer. The buffer contains (per liter) 20 g NH_4Cl, 10 g $NaH_2PO_4 \Sigma H_2O$, and 42.5 g $K_2HPO_4 \Sigma 3H_20$. This was shaken to homogenize the soil solution. Further serial dilutions of this were made into sterile nanopure water using an Eppendorf transfer pipette using sterile disposable tips.

The diluted soil solution was transferred in 0.1 ml aliquots to agar plates. A glass rod was used to spread the solution evenly across the surface of the plates. The plates were incubated for 4 days in the dark at 30∞C. These plates contained a rich source of nutrients in order to encourage growth of the soil microbes. The ingredients of the agar were (per liter) 800 ml nanopure water, 100 ml phosphate buffer (described above), 100 ml trace metals solution, 15 g agar, 5 g dextrose, and 0.5 g yeast extract. The metals solution contained (per liter) 0.03 g $ZnSO_4 \Sigma 7H_2O$, 0.03 g $MnSO_4 \Sigma H_2O$, 0.12 g $FeSO_4 \Sigma 7H_2O$, and 0.977 g $MgSO_4$. This was constantly mixed until homogenized and then autoclaved for 15 minutes at 250∞C. After autoclaving the solution was poured into plates, allowed to cool, and stored in the dark at 4.5∞C until needed.

After incubation the plates were counted. Colonies usually appeared as yellowish circular shapes on the surface of the plates. The number of colony forming units (CFU's) were counted for each plate. An important assumption here was that each CFU originated from a single organism. This was multiplied by a dilution factor equal to the inverse of the volume that the original 1 gram of soil was diluted by. Finally this was all divided by the dry soil mass diluted in order to express all counts as the number of organisms per dry gram of soil.

Benzoate Plate Counts

In addition to heterotrophic plate counts, which are an indicator of the overall health of the indigenous population, benzoate plate counts were also performed. Benzoate is a compound similar in structure to other aromatic petroleum constituents. In fact it is an intermediate in the metabolic pathway of some microorganisms. In general, benzoate is harder for microorganisms to degrade than dextrose, the compound used as a substrate in the heterotrophic plate counts. Therefore benzoate plate counts would be expected to be a more selective means of establishing the capability of indigenous soil microorganisms to degrade petroleum hydrocarbons than would heterotrophic plate counts.

The media used for the benzoate plate counts contained the same ingredients as the media used in the heterotrophic plate counts except that no dextrose was added. Instead, 1 g/l benzoate (supplied as sodium benzoate) was added. The plating procedure was identical as well.

Microcosms

In addition to the benzoate plate counts, microcosm studies were performed to provide further evidence of the petroleum degrading ability of each INDOT soil sample. Approximately 10 g (dry weight) of each soil was added to sterile 25 ml glass vials. To this 1 ul toluene was added, resulting in a toluene concentration of about 75 mg toluene per kg soil. This was immediately capped with a Teflon-lined rubber stopper and crimp sealed with an aluminum cap. An initial headspace sample of 500 ul was taken with a gas tight syringe and analyzed on the gas chromatograph. The oven program for the GC was constant at 120∞C for 8 minutes with the inlet at 123∞C and the detector at 210∞C. Further headspace samples (also 500 ul) were taken at regular intervals up through 13 days of testing for some soils. For other soils testing lasted only 5 days. Between sampling, all vials (microcosms) were incubated in the dark at 30∞C.

To ensure that toluene disappearance was of a biotic nature, controls were run. Soil samples (10 g) from each site were autoclaved for 15 minutes at 250∞C two times to sterilize the soil before adding toluene. Identical headspace testing was then performed. In addition to the sterilized controls, vials with only 1 ul toluene (no soil) were also incubated and toluene headspace was measured at 0 and 11 days.

Analysis of Data

Gasoline Standards

Unleaded gasoline standards were analyzed on the GC in order to compare extracts prepared from all INDOT soils. The response of the GC, measured in peak area, is proportional to the amount of product analyzed. The average area of each peak in the methanol blank was individually subtracted from the average area of each peak in the standards. An unleaded gasoline calibration curve with an intercept of 0.0994 was created having a correlation coefficient of 0.999994.

Soil Extractions, Headspace Testing, and Odor Testing

Calculations for the soil extractions were performed identically to those for the unleaded gasoline standards. However the concentration arrived at was only the concentration for the extract. This concentration was then multiplied by a dilution factor and divided by the dry soil mass to reach the soil total petroleum (TPH) concentration in mg TPH/kg soil, or ppm. With the exception of RDG1 (4930 ppm), all TPH concentrations were below 90 ppm (Table 1.). A statistical analysis of these extractions was performed to determine if the peak areas contributing to the TPH concentration were significantly different from the peak areas in methanol blanks. For each extract the area of the largest contributing peak was compared to the corresponding peak area

Table 1. Bacterial Populations, Toluene Biodegradation Rates and Total Petroleum Hydrocarbon Concentrations.

Site	Heterotrophic Plate Count CFU/g dry soil	Benzoate Plate Count CFU/g dry soil	Toluene Half-life (Days)	TPH (ppm)
Anderson 1	3,040,000	392,000	2	0
Anderson 2	2,590,000	543,000	1.5	0
Bean Blossom 1	4,140,000	978,000	2	2.92
Bean Blossom 2	2,440,000	582,000	1.25	0
Dale1	10,600,000	1,040,000	2.25	11.6
Dale2	2,160,000	721,000	2	38.4
Hamlet 1	2,870,000	1,830,000	1.25	17.3
Hamlet 2	2,180,000	1,140,000	1.5	17.4
Hanna 1	5,520,000	2,140,000	1.5	39.7
Hanna 2	5,540,000	1,650,000	1	56.9
Ridgeville 1	1,850,000	488,000	1.5	4930
Ridgeville 2	4,150,000	1,400,000	1	83.4
St Johns 1	3,950,000	255,000	1.5	0
St Johns 2	5,440,000	1,000,000	1	0
Vincennes 1A	4,010,000	2,260,000	5	30.4
Vincennes 1B	13,500,000	1,430,000	5	21.9
Vincennes 2	4,300,000	967,000	2	0

in the methanol blank using a t-test at the 90% confidence interval. In cases where peaks appeared in extracts that did not appear in the blanks, significance was assumed. Five out of 6 samples having TPH concentrations greater than 30 ppm were significant at the 95% confidence interval, 3 of them by virtue of peaks exclusive to their chromatograms. Five out of 11 samples with TPH concentrations below 30 ppm were significant at the 90% confidence interval (only 1 by virtue of a peak exclusive to its own chromatogram), 3 of these were significant at the 95% confidence interval.

Heterotrophic Plate Counts

In all samples a thriving microbial heterotrophic population was found to exist. Populations ranged between 1,850,000 CFU/g soil and 13,500,000 CFU/g soil (Table 1.). Of interest here is that the sample with the lowest heterotrophic population, Ridgeville1, also had the highest TPH measurement. Excessively high TPH concentrations can be toxic to microorganisms, preventing biodegradation. But when heterotrophic plate counts were plotted against hydrocarbon concentrations no apparent correlation existed (Figure 1). This is not altogether surprising as a high concentration of hydrocarbons alone is not enough to retard all microbial growth: the mixed microbial population can survive on other substrates such as naturally occurring organic matter. Conversely, a high concentration could provide an abundance of food for a soil microbial population but the correlation does not bear this out either: microbes need other factors, such as oxygen, water, and nutrients to thrive and multiply. So the lack of a direct correlation between heterotrophic plate counts and hydrocarbon concentration is not surprising.

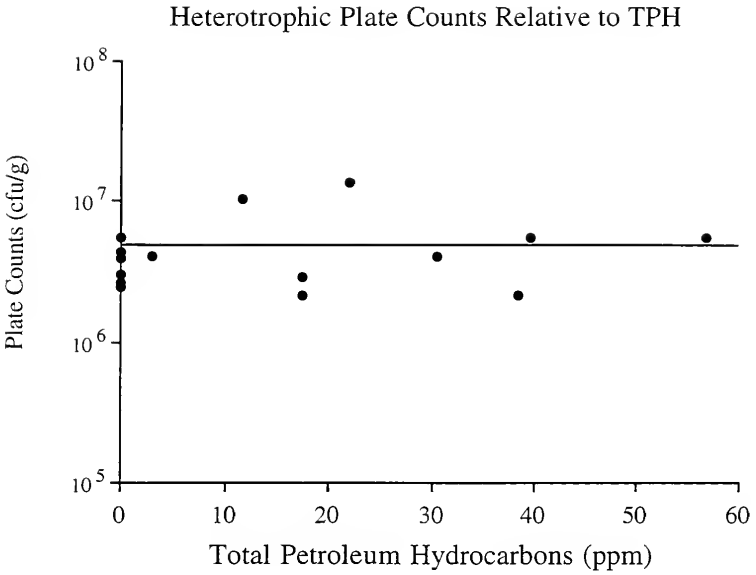

Figure 1.

Benzoate Plate Counts

While heterotrophic plate counts can provide a general idea of the health of the indigenous microbial population, it does not discriminate as to the catabolic capabilities of the soil microbes. Heterotrophic plate counts are run with dextrose, a simple sugar that is used by most bacteria. In order to provide insight as to the indigenous microbial population's ability to handle substrates akin to petroleum hydrocarbons, benzoate plate counts were performed. Benzoate is an intermediate in the degradation pathway of toluene and therefore is an ideal choice for a compound to provide an

indication of aromatic degrading capacity of a soil. Thus it should be obvious that a benzoate plate count should be lower than the heterotrophic plate count for the same sample: benzoate plate counts are more selective. The results confirm this claim with populations ranging between 255,000 CFU/g soil (St Johns 1) to 2,260,000 CFU/g soil (Vincennes 1A). Worthy of note is that St Johns 1, the site with the lowest benzoate plate count, did not have the lowest heterotrophic plate count, it had the 10th highest count. The site with the highest benzoate plate count, Vincennes 1A, had only the 9th highest heterotrophic plate count. Upon comparing the benzoate plate counts versus their corresponding heterotrophic plate counts, it was found that little relationship exists between the benzoate degrading population and the heterotrophic population (Figure 2). Ideally, though, the benzoate-degrading population would increase as the heterotroph population increased. In other words, the fraction of the microbial population capable of degrading benzoate in all cases would be nearly a constant. But due to different site conditions such as hydrocarbon concentration, water content, oxygen concentration, and nutrient availability, optimal conditions for benzoate-degrading bacteria are not present in all soils tested.

Attempts to draw an additional correlation, one between benzoate plate counts and hydrocarbon concentrations, were made as well (Figure 3). Here again no significant correlation was found. A positive correlation would imply that an increase in the hydrocarbon concentration would correspond to a increase in the benzoate-degrading population. Intuitively this makes some sense. These soils were sampled after an indeterminate time following the suspected fuel leaks. Therefore in the period between the fuel release and when sampling occurred, microbial degradation of some of the petroleum hydrocarbons could have occurred resulting in an increase in benzoate degraders.

One point of interest is that none of the correlations were attempted with nutrient supply as a variable. Nutrient availability was not limiting in the soils tested.

Populations of Benzoate Degraders Relative to Heterotrophic Plate Counts

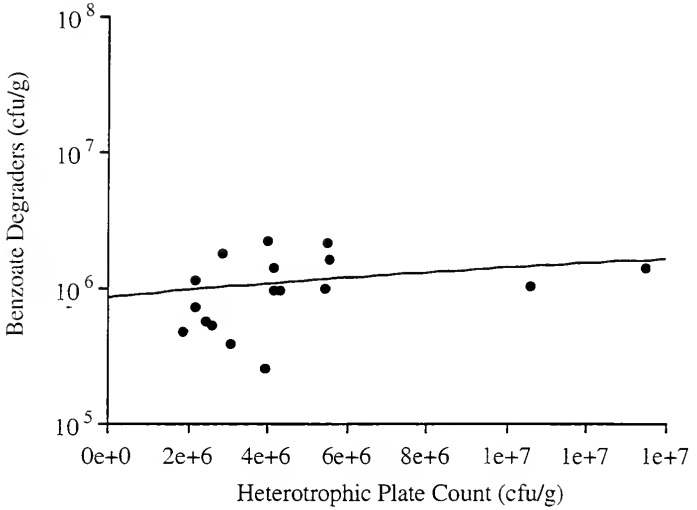

Figure 2

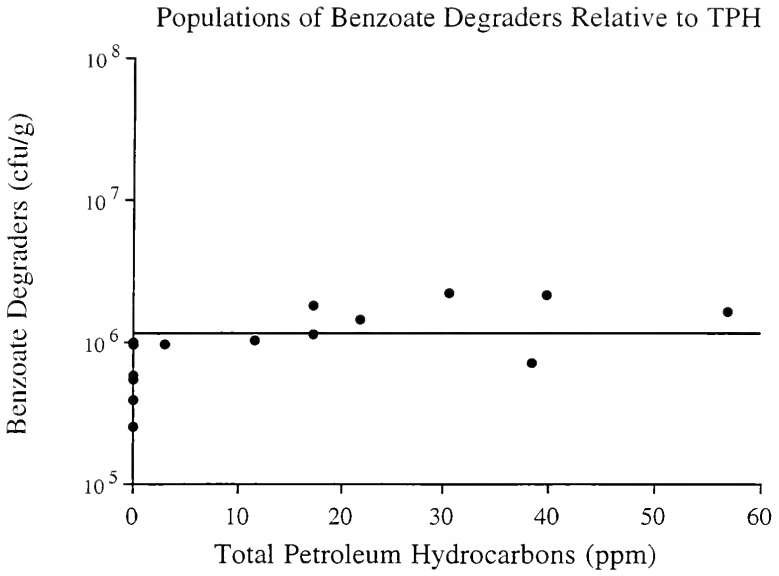
Populations of Benzoate Degraders Relative to TPH
Benzoate Degraders (cfu/g)
10^8
10^7
10^6
10^5
0
10
20
30
40
50
60
Total Petroleum Hydrocarbons (ppm)

Figure 3.

Microcosm Studies

Lacking any strong correlation between aforementioned tests, bench-scale microcosm studies were performed on all INDOT soils to assess the capabilities of indigenous soil microflora to degrade a model petroleum compound (toluene). With the exception of Ridgeville 1, all soils degraded greater than 90% of added toluene over a 4 day period. 6 of these soils degraded greater than 90% of added toluene in 2 days and 5 of them degraded greater than 95% of added toluene in 2 days. In all cases, after the toluene had been degraded the samples were respiked with toluene and again the toluene was degraded.

One factor which did seem to affect toluene biodegradation rates in the soil microcosms was soil type (Figure 4). Clay soils seem to inhibit biodegradation rates of toluene by about a factor of 2. Whether this was due to reduced soil porosity or some other factor is unknown. Combining this obsrvation with the fact that microbial populations of heterotrophs and benzoate degraders were relatively constant, 10^7 and 10^6 cfu/g respectively, one could propose that physical parameters have the greatest influence on biodegradation.

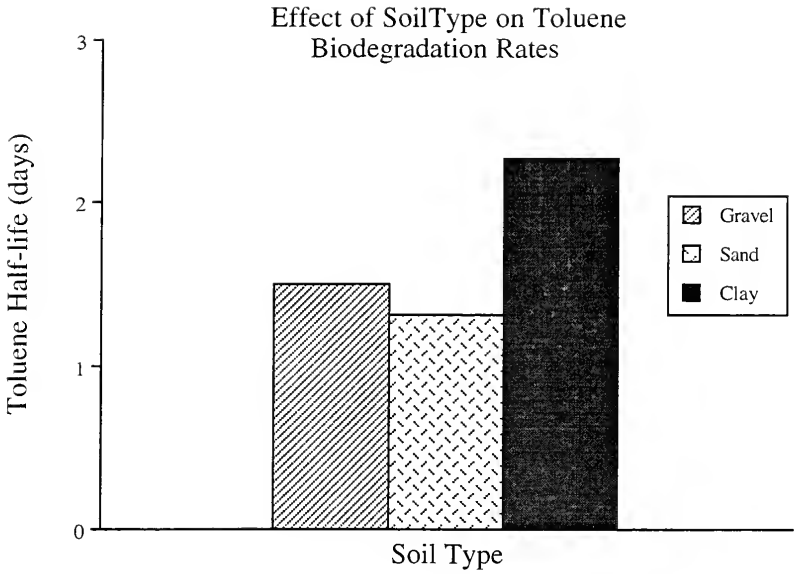
Effect of SoilType on Toluene
Biodegradation Rates
Toluene Half-life (days)
3
2
1
0
Soil Type
Gravel
Sand
Clay

Figure 4.

Conclusions

All INDOT soils tested had three things in common. First, a thriving heterotrophic population existed. Second, bacteria capable of degrading benzoate was present in all soils. However, neither of these two parameters correlated well to the total petroleum hydrocarbon concentration present in the soil. Finally, toluene biodegradation in all soils indicated indigenous populations with the catabolic capabilities to remediate petroleum hydrocarbons. This study provides some evidence that physical rather than microbial parameters control biological processes in soil. Based on these results as a whole, we can firmly conclude that the potential for in-situ bioremediation of petroleum hydrocarbons exists.

Recommendations

Bioremediation is often the most cost-effective and successful technique available for the remediation of soils and groundwater contaminated with organic pollutants (e.g. petroleum). Therefore, it is recommended that steps be taken to increase utilization of bioremediation for the remediation of petroleum contaminated soils and groundwater. Leaking underground storage tanks have resulted in petroleum contaminated soils at many INDOT facilities and these sites now require remediation. Contractors implementing bioremediation must be certain that their design is justified by sound science and engineering principles. However, there are no currently existing "design codes" or "standard practices" for the design, implementation or monitoring of bioremediation projects. For example, some bioremediation strategies are only marginally justified by the research literature, and often not justified at all by the contractor's own data. In addition, many soils in Indiana have low hydraulic conductivities which increases the difficulty of implementing successful bioremediation projects. Low hydraulic conductivities of soil is one of the factors

which has been identified by the National Research Council as limiting the potential applicability of bioremediation with our current state of knowledge.

Landfarming has been used successfully to treat petroleum contaminated soils and may br applicable for soils with low hydraulic conductivities. Landfarming is a desirable treatment option when in-situ treatment is not possible, for example, when contaminated materials are encountered during construction projects. Several landfarming methods exist, however each method avoids the ultimate use of landfills for soil disposal. Treatment processes range from the use of traditional farm equipment to till and aerate contaminated soil to constructed biotreatment reactors. One of the most economical and effective landfarming methods used for treating petroleum contaminated soils is composting. Woodchips, manure, and contaminated soil are mixed into piles or windrows. The woodchips provide bulk and promote oxygen transfer for aerobic biodegradation and the manure provides nutrients for the microorganisms. When petroleum constituents are biodegraded the compost can be used as top-soil or backfill material.

Implementation Suggestions

In general, to increase utilization of bioremediation it is recommended that INDOT implement field demonstration projects. In conjunction with these projects several tasks are proposed:

- Develop a guidance manual for use by INDOT personnel and contractors. The manual will describe procedures for determining when implementation of bioremediation is appropriate, selection of rational bioremediation strategies, and will provide standards for good practice.
- Investigate methods for bioremediating soils with low hydraulic conductivities. Land farming offers the most attractive and potentially successful approach.

Bioremediation implementation will result in more effective site remediation and significant cost savings to INDOT through application of bioremediation where appropriate. Development of "standard practices" for bioremediation will reduce uncertainties when dealing with contractors and setting performance criteria. Developing techniques for expanding bioremediation application to low permeability soils will result in additional cost savings for INDOT site remediation.

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